FURTHER STUDIES ON GENTIAN VIOLET AS A MEANS OF ELIMINATING SPURIOUS PRESUMPTIVE TESTS FOR B. COLI IN WATER*

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The authors' first series of tests showed that the addition of 1–100,000 gentian violet to lactose broth inhibited 94.5 per cent of the spurious presumptive tests for B. coli caused by non-significant gas forming anaerobes (and aerobes) in water. Increasing the concentration to 1–20,000 only raised the efficacy of inhibition, in the case of samples heated to eliminate non-sporulating organisms, to 95 per cent. As to unheated samples, 75 and 81.8 per cent yielded B. coli with gentian violet as against 57.1 and 59.1 per cent without the dye, as shown in the accompanying data abstracted from a former paper.[1] The authors were later definitely able to raise the

	POSITIVE PRESUMPTIVE TESTS	B. COLI	PER CENT
Standard test	21	12	57.1
Gentian violet, 1–100,000	20	15	75.0
Standard test	44	26	59.1
Gentian violet 1–20,000	33	27	81.8

percentage of successful isolations of B. coli through the use of 1–100,000 gentian violet in the plating medium to inhibit aerobic spores, as shown in the following, also taken from the former paper.[1]

	POSITIVE PRESUMPTIVE TESTS	B. COLI ISOLATED	PER CENT
Standard test	22	20	90.9
Gentian violet 1–20,000	21	19	90.5

*The use of gentian violet to eliminate spurious presumptive tests for B. coli in water was discussed by these authors in the *Journal of Bacteriology*, July, 1918. The numerical references in the paper are to the bibliography at the end.

This is fairly satisfactory since B. coli can not be anticipated in every positive presumptive test at this concentration of gentian violet, owing to the fact already shown by the authors that certain few anaerobes are not inhibited even by a concentration of 1–10,000 gentian violet in the media.

The authors had hoped, however, to reach 100 per cent efficiency in isolation of B. coli from positive presumptive tests through increasing the concentration of gentian violet. The present paper points out the realization of this, but also notes the inhibition of the colon bacillus itself at concentrations sufficiently high to inhibit all other gas-forming organisms. In attempting to avoid completely spurious positive presumptive tests, the difficulty of false negative presumptive tests has been encountered. The results have involved the examination of forty samples of water with varying concentrations of gentian violet in the lactose broth presumptive test. These samples were selected by Mr. Frank Bachman of the Bureau of Sanitary Engineering of the California State Board of Health from routine water examinations yielding positive presumptive tests. The authors express their indebtedness for this courtesy. That they have obtained negative results in some cases with samples originally positive is due to delay intervening between the respective tests and consequent loss of gas-forming organisms.

Double strength broth was prepared containing 2 per cent lactose, 2 per cent Difco peptone, and 1 per cent NaCl in meat infusion. The reaction was titrated to 1 per cent normal acidity. This was divided into four lots, to which were added sufficient Grübler's gentian violet to give concentrations of 0, 1–4500,—1, 1500, and 1–500, respectively. The media were tubed in Durham fermentation tubes and sterilized in an Arnold sterilizer by the intermittent method. All other media used were standard, except the litmus lactose plating agar, which contained an addition of 1–100,000 gentian violet for the reasons mentioned in the authors' first paper.

Water samples for the presumptive test were thoroughly shaken. Ten cubic centimeters were then added to each tube so that the final concentrations of ingredients were 1 per cent lactose, 1 per cent peptone, 0.5 per cent NaCl and 0, 1–9000, 1–3000, and 1–1000, respectively, of gentian violet. The tests were incubated at 37°C. and examined daily for gas production up to five days.

Upon the appearance of gas the lactose broth culture was streaked upon one-half the surface of a gentian violet lactose agar plate.

(These plates are best prepared the day before by pouring sterile media into sterile Petri dishes with tile covers to absorb excess moisture. Drying over night at room temperature minimizes spreading, but excessive evaporation after twenty-four hours must be prevented, e.g., by exchanging the tile cover for a sterile glass cover.) The streaked plates were examined on twenty-four and forty-eight hour incubation at 37°C. for acid colonies. If acid colonies appeared, or if, in case none appeared, the colonies resembled B. coli, they were picked to the unused half of the plate. At the end of another twenty-four hours incubation, Gram stains were made and Gram-negative, non-sporulating bacilli confirmed, if possible, as colon bacilli by the usual tests on lactose broth and gelatin at 37°C.

In this procedure the authors have somewhat exceeded the prescription of the Standard Methods for the Examination of Water and Sewage of the American Public Health Association for 1917 in the matter of time of incubation and in the addition of the gelatin test. Also, as in their first series,[1] a second trial was made, if the first failed to yield B. coli, by subculturing from the original presumptive test to a new tube of similar media, and so repeating.

After the examination of 14 samples the method was modified in that the criterion for plating a sample was taken to be either the production of gas or the appearance of growth alone in the presumptive test, as determined by comparing the turbidity of the inoculated tube with an uninoculated tube of the same dilution of dye. Following this technic 26 more samples were examined. Inspection of the original data, however, shows that in but two instances were colon bacilli isolated from presumptive tests showing growth only (which was delayed till the third day), once from a dilution of gentian violet of 1–3000 and once from a dilution of 1–1000.

The first appearance of gas in the presumptive tests of this series is shown in table 1. The summary of the complete data is made as if gas were, as usual, the only criterion for plating, eliminating the two cases above mentioned from table 2.

The data of table 1 support the authors' earlier findings that we are justified in waiting at least four days before calling a presumptive test negative. There is no question that increased concentration of gentian violet in the lactose broth presumptive test markedly delays gas production by the colon bacillus.

It is apparent from table 2 that the formation of gas in seven of the above samples must be attributed to organisms other than

colon bacilli. The relatively large proportion of non-acid plates from which B. coli was isolated is at once disappointing and instructive; it is distinctly higher than in the author's first series.[1] Such plates are frequently to be explained by the co-existence of highly proteolytic bacteria which neutralize the acid produced by colon

TABLE 1

First appearance of gas in presumptive tests in 40 samples of water tested for gas production in varying dilutions of gentian violet lactose broth

DYE	NUMBER OF SAMPLES WITH B. COLI ISOLATED						NUMBER OF SAMPLES WITH B. COLI NOT ISOLATED		
	1 day	2 days	3 days	4 days	5 days	Total	1 day	2 days	Total
0	20	8	2			30	3	4	7
1–9000	8	13	3	1		25			
1–3000		8	5	1		14			
1–1000					1	1			

TABLE 2

Isolation of B. coli from 40 samples of water tested for gas production in varying dilutions of gentian violet lactose broth

PRESUMPTIVE TEST			ACID PLATES	SAMPLES FROM WHICH B. COLI WAS ISOLATED AND IDENTIFIED				
Dye	Gas	Growth only		1st trial	2nd trial	Total	Ratio "a"	Ratio "b"
0	37	1	21	27	3	30	0.818	1.000
1–9000	25	10	12	23	2	25	1.000	0.833
1–3000	14	12	10	15	1	14	1.000	0.466
1–1000	1	7	1	1	0	1	1.000	0.033

$$\text{Ratio a} = \frac{\text{Colon samples at given concentrations of gentian violet}}{\text{Samples showing gas in presumptive test at given concentration of gentian violet}}$$

$$\text{Ratio b} = \frac{\text{Colon samples at given concentration of gentian violet}}{\text{Highest number of colon samples at any concentration of gentian violet of the series}}$$

bacilli; most of these organisms being non-lactolytic, alkali production proceeds from the beginning of their growth on the plate. Unfortunately, they are among the worst spreaders and being Gram-negative are not inhibited by gentian violet as are the Gram-positive sporulating spreaders of the hay bacillus group. From such plates B. proteus, B. pyocyaneus and B. fluorescens are frequently obtained.

Another explanation of plates containing B. coli failing to show acid colonies is that the organisms have exhausted the sugar and begun their attack upon the protein of the media with resultant alkali formation. It is true that reversion from the acid phase to the alkaline phase can be delayed by increasing the lactose content from 1 per cent to 2 per cent, but there need be no trouble from this source alone if the plates are examined on twenty-four hours incubation. For most strains of colon bacilli 1 per cent lactose is in excess of their fermentative capacity unless some means is taken to prevent the accumulation of acid.

The further difficulty of "attenuated" B. coli presents no adequate explanation or solution.

The use of gentian violet in slightly increased concentration in the presumptive test excludes the production of gas by samples from which B. coli can not be isolated, but especially in somewhat greater concentration prevents the production of gas by certain samples in which B. coli is unquestionably present. A dilution of 1–1000 gentian violet almost completely inhibits growth and gas production. In most instances growth without gas formation in the presence of gentian violet has been found to be due to B. proteus and other gelatin liquefying, Gram-negative, non-sporulating microorganisms. These appear to be somewhat more resistant to gentian violet than B. coli.

The upshot of the matter is, therefore, that gentian violet can not be used in as strong a concentration as 1–9000 in the lactose broth presumptive test without danger of inhibiting some colon bacilli; something near this is necessary to inhibit all spurious tests, however. 1–20,000 gentian violet, on the other hand, not only increases the total number of samples from which B. coli can be isolated, but reduces the number of spurious presumptive tests to a minimum.

The condition can perhaps be best displayed graphically by computing the ratios,

$$\text{(a)}\quad \frac{\text{Colon samples at given concentration of gentian violet}}{\text{Samples showing gas in presumptive test at given concentration of gentian violet.}}$$

and

$$\text{(b)}\quad \frac{\text{Colon samples at given concentration of gentian violet}}{\text{Highest number of colon samples at any concentration of gentian violet of the series.}}$$

from the average data of tables 2, 3 and 4 and 5 of the author's first paper[1] and table 2 of the present communication, and plotting these against the logarithm of the dilution as in figure 1. The data so plotted are shown in table 3.

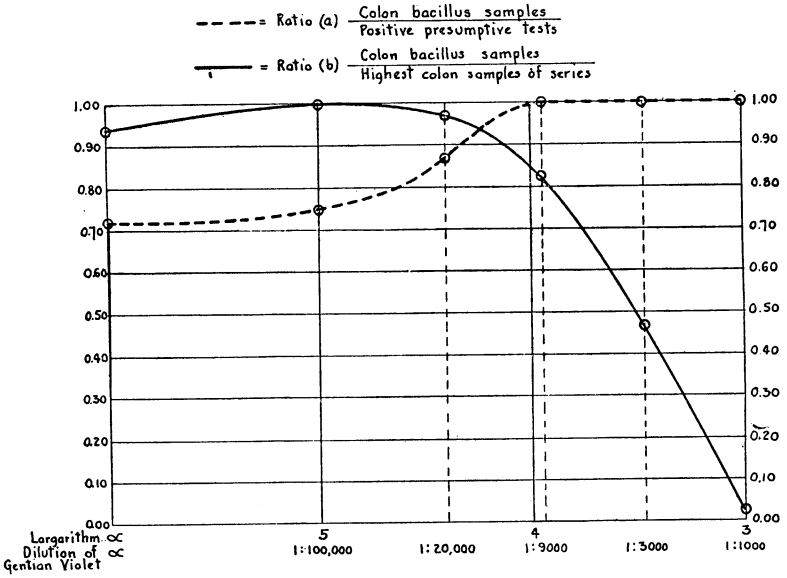

FIG. 1

TABLE 3

DILUTION* OF GENTIAN VIOLET	LOGARITHM OF THE DILUTION	RATIO	
		(a)	(b)
∝	∝	0.725	0.941
100,000	5.00	0.750	1.000
20,000	4.30	0.861	0.975
9,000	3.95	1.000	0.833
3,000	3.48	1.000	0.466
1,000	3.00	1.000	0.033

*Number of cubic centimeters of broth in which 1 gram of gentian violet would be found.

The curves obtained do not coincide in any part. Where they cross between a dilution of 1–20,000 and 1–9000 certainly indicates the maximum concentration which should be used; the optimum lies between 1–20,000 and 1–100,000, probably nearer 1–100,000,

but some spurious presumptive tests must be expected at this concentration. Either of these concentrations can be relied upon to give results superior to the standard test, however.

It is interesting to note that the number of colon bacilli implanted in lactose broth is a factor in the inhibition of gas production by gentian violet; inhibition occurs only when the number of organisms implanted is small. Meat infusion broth with 1 per cent Difco peptone, 0.5 per cent NaCl, 1 per cent lactose and 1–1000 gentian violet was sterilized in Durham fermentation tubes, 9 cubic centimeters each. The control set of media was identical except for the dye. From a twenty-four hour broth culture of B. coli, dilutions in sterile 0.85 per cent NaCl were made, ranging from 1–10 (10^{-1}), 1–100 (10^{-2}), 1–1000 (10^{-3}), etc., to 10^{-20}. One cubic centimeter of each dilution was added to a culture tube containing 9 cc. of the lactose broth. The tubes were incubated at 37°C. and examined for gas daily for three days. From each tube showing gas there was subcultured on plain agar a characteristic coliform bacillus. The results are shown in table 4.

It is most important to record that similar tests with glucose broth failed to show any such inhibition by gentian violet in the same concentration. This point is shown in table 5, which is the record of a carefully controlled experiment using the same culture dilutions for implantation of four sets of media as follows—glucose broth with and without gentian violet, and lactose broth with and without gentian violet. In these tests the media were sterilized in the autoclave before the addition of carbohydrates which were sterilized separately by autoclave in 10 per cent solution in neutral distilled water, and added aseptically in proper proportion to make 1 per cent solutions in the broth. Autoclave sterilization of 10 per cent solution of carbohydrates in neutral distilled water gives no evidence of hydrolysis and avoids the difficulties recently emphasized by Mudge[2] and frequently encountered by every bacteriologist in the sterilization of sugar media. We feel that the above procedure is preferable either to filtration as advocated by Mudge, or to a short sterilization at high temperature as prescribed by the 1917 Standard Methods. The latter especially has been seriously criticized by Hasseltine[3] as inferior to intermittent sterilization in the Arnold sterilizer.

Similar results were obtained repeatedly with meat infusion broth. Of various interpretations which suggest themselves, a

plausible one is that in the presence of glucose the dye is chemically bound so that it becomes less active in the presence of even a small number of organisms. These results might appear to afford a basis for the assumption of carbohydrate-dye compounds analogous to the protein-dye compounds, of which there can be little doubt if we are to accept the conclusions of such comprehensive

TABLE 4

Dependence of inhibition of gas production by B. coli in lactose broth upon the small number of organisms inoculated

DILUTION OF CULTURE	NO GENTIAN VIOLET	GENTIAN VIOLET 1-1000		
	24 hours	24 hours	48 hours	72 hours
10^{-1}	0	0	0	0
10^{-2}	0	0 trace	0	0
10^{-3}	0	0 trace	0	0
10^{-4}	0	—	0	0
10^{-5}	0	—	0	0
10^{-6}	0	—	0 Trace	0
10^{-7}	0	—	0 Trace	0
10^{-8}	0	—	0 Trace	0
10^{-9}	0	—	0	0
10^{-10}	0	—	0	0
10^{-11}	0	—	—	0
10^{-12}	0	—	—	0
10^{-13}	0	—	—	0
10^{-14}	0	—	—	0
10^{-15}	0	—	—	0
10^{-16}	0	—	—	0
10^{-17}	0	—	—	0
10^{-18}	0	—	—	0
10^{-19}	0	—	—	0
10^{-20}	0	—	—	—*

*Positive in five days. 0 indicates gas production.

The essentials of the above data were confirmed by repetition of the experiment.

reviews and investigations as those of Mathews,[4] Heidenhain,[5] Mann[6] and Robertson,[7] on this subject. A single failure to repeat the results of table 5 with meat extract broth in which no growth of B. coli could be obtained in either plain, glucose, or lactose broth with 1–1000 gentian violet may be taken to indicate that protein enters into the reaction, possibly in the form of glucosamin.

We may, therefore speculate somewhat as follows: lactose, having no such ability as glucose of combining with gentian violet, leaves the dye free to act upon the bacteria in such a way as to prevent their multiplication. There is no evidence to show that inhibition of gas formation occurs without inhibition of growth, otherwise we might conclude that the sugar is rendered unavailable. In either case their is no noticeable precipitate in the medium such as is frequently the case in dye protein compounds.

The introduction of large numbers of B. coli appears to overrule the inhibitive action of gentian violet in a dilution of 1–1000, and a *delayed* growth and gas production may occur with relatively small

TABLE 5

Failure of inhibition of gas production by B. coli in glucose broth

DILUTION OF CULTURE	GLUCOSE BROTH		LACTOSE BROTH			
	No gentian violet 24 hours	Gentian violet 1–1000 24 hours	No gentian violet 24 hours	Gentian violet 1–1000		
				24 hours	48 hours	72 hours
10^{-1}	0	0	0	0	0	0
10^{-2}	0	0	0	0	0	0
10^{-3}	0	0	0	Trace	0	0
10^{-4}	0	0	0	Trace	0	0
10^{-5}	0	0	0	—	Trace	0
10^{-6}	0	0	0	—	Trace	Trace
10^{-7}	0	0	0	—	Trace	Trace
10^{-8}	0	0	0	—	Trace	Trace
10^{-9}	0	0	0	—	—	Trace
10^{-10}	0	0	0	—	—	—
10^{-11}	—	—	0	—	—	—
10^{-12}	—	—	—	—	—	—

0 indicates gas production.

numbers of introduced bacteria. These phenomena might be interpreted as due either to acclimation of the bacteria to the dye, for which Shiga[8], and Fitzgerald and Mackintosh[9], have separately presented evidence in the case of other organisms, or to reduction of germicidal power by adsorption between certain individual bacteria and the dye, leaving certain other individuals free to multiply.

Analogous to the action of lactose is that of agar, which belongs to the group of colloidal polysaccharids, in the presence of which

both Gram-positive anaerobes, such as B. oedematis, B. botulinus, and B. welchii, and Gram-negative aerobes, such as B. coli, require more gentian violet for their inhibition.

SUMMARY

This paper extends the observation previously made showing the practical utility of a high dilution of gentian violet for eliminating spurious presumptive tests for B. coli in the lactose broth fermentation test of polluted water. It is shown that if the concentration of dye be sufficiently increased every positive presumptive test can be relied upon to yield B. coli, but the total number of positive tests is likely to be reduced. A low concentration of dye, however, yields a higher proportion of successful isolations of B. coli than the standard method, yet a certain low percentage of spurious presumptive tests must be anticipated.

Glucose broth containing a high concentration of gentian violet is less inhibitive for B. coli than lactose broth, which leads to certain theoretical considerations involving the possibility of dye carbohydrate compounds analogous to dye protein compounds.

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